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2/ROT3<sup>1</sup>

## Gene participating in the synthesis of brassinosteroid

Field of the invention:

Present invention relates to a gene participating in the synthesis of  
5 brassinosteroid, more specifically, to a novel gene (CYP90D1, SEQ ID NO: 1)  
controlling the final step of synthesis of brassinosteroid in combination with ROT3  
gene (=CYP90C1, #51 to #1625 of SEQ ID NO: 3).

Prior Art:

10 Brassinosteroids are a kind of plant hormones, which are ubiquitously  
distributed throughout the plant kingdom and are functional in cell elongation and  
cell division at extremely low concentrations, and are generic name of more than 40  
kinds of analogues.

15 Because of their strong action on plants, brassinosteroids have been suggested to  
be important in their applicability to agricultural industry and many patents related  
to them have been disclosed (e.g., Japanese Unexamined Patent Publications,  
5-222090, 6-98648, 6-340689, 8-59408, 8-81310, 8-113503, 9-97).

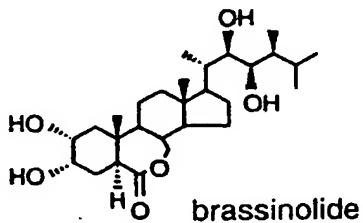
20 Researches on brassinosteroid biosynthesis have been made aggressively and the  
progressed elucidation of biosynthetic pathways (e.g., Fujioka et al., "Brassinosteroid  
biosynthesis and signal transduction" in Signal Transduction of Plant Hormones  
p180-189, Cell Technology Supplement, Plant Cell Technology Series 10, Shujunsha  
Co. Ltd, (1998)) suggests that cytochrome P450-type proteins regulate the  
brassinosteroid biosynthesis in plant.

25 The inventors already identified ROTUNDIFOLIA3 (ROT3) gene, which belongs  
to a family of cytochromes P450, in *Arabidopsis* (Gene & Development 12:2381-2391  
(1998)), and showed that modulation of the expression of ROT3 gene resulted in  
morphological alterations of leaves and flowers (Proc. Natl. Acad. Sci. USA vol. 96, pp.  
9433-9437 (1999)).

30 Problems to be solved by the invention:

As described above, nucleic acid molecules encoding cytochrome P450-type  
proteins, which are involved in brassinosteroid biosynthesis, have been identified

(published Japanese translation of PCT international publication for patent application (WO97/35986) No. 2000-508524). However, nucleic acid molecules previously known for the biosynthetic pathway are involved in regulation of the steps in comparatively early stage in brassinosteroid biosynthesis. Therefore, it was 5 difficult to apply the action of the above-described nucleic acid molecules to the organ specific control or to the quantitative regulation. Furthermore, neither the enzyme proteins nor nucleic acids encoding the proteins, regulating the final step of brassinosteroid biosynthesis, have been known. The final step as used herein means the step to synthesize brassinolide (the formula described below) from castasterone 10 (The whole synthetic pathway of brassinosteroid is shown in Figure 1).



Means to solve the problems and Detailed description of the Invention:

The inventors searched homological nucleotide sequences to ROT3, which the inventors had previously discovered, and found a nucleotide sequence that exhibits 15 51% identity to ROT3 gene. Examining the sequence, the inventors discovered that the sequence is a novel gene (CYP90D1, SEQ ID NO: 1), which encodes a factor regulating the final step of the brassinosteroid biosynthesis, physiologically functional in regulating the size of plant. Furthermore, the inventors discovered that the CYP90D1 gene regulates the final step of the brassinosteroid biosynthesis in 20 combination with ROT3 (=CYP90C1) gene, then accomplished the present invention.

The present invention makes it possible to regulate the biosynthetic pathway of physiologically active brassinosteroid using ROT3 (=CYP90C1) and CYP90D1 and this possibility of regulation is the unique point that differentiates the invention from previous findings.

In other words, the expression of sole ROT3 (=CYP90C1) in a whole plant is effective only to leaves and floral organs, particularly effective in longitudinal direction. Floral organ are derived from deformed leaves, there are common pathways between them in morphological regulation by genes. On the other hand, 25

the combination of ROT3 (=CYP90C1) and CYP90D1 is effective to a whole plant. By manipulating nucleic acid molecules of ROT3 (=CYP90C1) and CYP90D1 and proteins coded by these genes, the shape of leaves and flowers can be changed at will and at the same time, and also only the shape of flowers can be changed without changing 5 the major part of the height of plants and the shape of leaves.

That is to say, the present invention is a gene (A) having the nucleotide sequence of (1) or (2):

- (1) Nucleotide sequence of SEQ ID NO: 1.
- (2) Nucleotide sequences encoding either of the following proteins,

10                     (a) A protein having the amino acid sequence of SEQ ID NO: 2.  
                       (b) A protein having the amino acid sequence derived from SEQ ID NO: 2,  
wherein one or some amino acids are deleted, substituted or added and its expression  
stimulates brassinosteroid biosynthesis.

Furthermore, the present invention is also a polynucleotide (B) having the  
15 nucleotide sequence of (1) or (2), and that of (3) or (4):

- (1) Nucleotide sequence of SEQ ID NO: 1.
- (2) Nucleotide sequences encoding either of the following proteins,
  - (a) A protein having the amino acid sequence of SEQ ID NO: 2.
  - (b) A protein having the amino acid of SEQ ID No: 2, wherein one or some amino  
20 acids are deleted, substituted or added and its expression stimulates brassinosteroid  
biosynthesis.
- (3) Nucleotide sequence of #51 to #1625 of SEQ ID NO: 3.
- (4) Nucleotide sequence encoding either of the following proteins,
  - (c) A protein having the amino acid sequence of SEQ ID NO: 4.
  - (d) A protein having the amino acid sequence of SEQ ID NO: 4, wherein one or  
some amino acids are deleted, substituted or added and its expression stimulates  
brassinosteroid biosynthesis.

Moreover, the present invention is i) a polynucleotide comprising a promoter and  
the gene (A), whose nucleotide sequence is linked to said promoter in forward  
30 direction, ii) a polynucleotide comprising a promoter and the gene or a part of the  
gene (A), whose nucleotide sequence or a part of the sequence is linked to said  
promoter in reverse direction, iii) a polynucleotide comprising a promoter and the

polynucleotide (B), wherein both of the above-described nucleotide sequences are linked to said promoter in forward direction, or iv) a polynucleotide comprising a promoter and the polynucleotide (B) or a part of them, wherein at least one of nucleotide sequence of the above-described nucleotides or a part of them is linked to  
5 the above-described promoter in reverse direction.

The promoter used herein will be described later in detail and includes the cauliflower mosaic virus 35S promoter, heat shock promoter, chemical-inducible promoters and others. Additionally, there are no limits on the way to link a promoter with the above-described gene and the linking can be operated appropriately using  
10 conventional techniques of genetic engineering.

Still furthermore, the present invention is a plasmid containing either of the above-described genes or the above-described polynucleotides and is also a plant transformed by either of the above-described genes or the above-described polynucleotides.

15 Still moreover, the present invention is a plasmid containing the above-described polynucleotide. The plasmids used herein include such binary vectors as pBI-121 plasmid, Ti plasmid and others.

Also, the plants applicable by the present invention cover whole Spermatophyte.

To transform such plants, the gene of the present invention was inserted to the  
20 above-described plasmid, which may transform the above-described plants using conventional genetic engineering methods.

In addition, the present invention is a method for altering the morphology of a plant, comprising the steps of transforming a plant by the gene (A) or by the polynucleotide (B) and enhancing or suppressing the expression of the  
25 above-described gene or the above-described polynucleotide. Furthermore, the present invention is a method for altering the morphology of a plant, which is transformed by any of the above-described genes or polunucleotides, comprising the step of stimulating the responsible promoter in the transformed plant. And also, the present invention is the plant with a morphology altered by any of the  
30 above-described methods.

Also, the present invention is a protein of the following (a) or (b):

(a) A protein having the amino acid of SEQ ID NO: 2.

(b) A protein having the amino acid sequence of SEQ ID NO: 2, wherein one or some amino acids are deleted, substituted or added and its expression stimulates brassinosteroid biosynthesis.

Furthermore, the present invention is a mixture or a complex of the  
5 above-described protein and a protein of the following (c) or (d):

(c) A protein having the amino acid of SEQ ID NO: 4.

(d) A protein having the amino acid sequence of SEQ ID NO: 4, wherein one or some amino acids are deleted, substituted or added and its expression stimulates brassinosteroid biosynthesis.

10 It is possible to manipulate nucleic acid molecules of CYP90D1 and ROT3 (=CYP90C1) and proteins coded by these using the following procedures:

(1) The procedure comprises the steps of linking a manipulable promoter with DNA molecules of CYP90D1 (SEQ ID NO: 1) and ROT3 (=CYP90C1, #51 to #1625 of SEQ ID NO: 3), transducing these into a plant by way of an appropriate conventional method such as Ti plasmid, and giving external stimulation to the promoter to regulate the expression of the above-described genes. The examples of the promoters usable herein are as follows:

\* A 35S promoter (possible to express constitutively)

\* A heat shock promoter (possible to express temperature dependently)

20 \* A Dex-inducible promoter (possible to express by the exposure to Dexamethason)]

\* A CHS-A promoter of petunia (petal specific expression in plants with colored petals and sugar-dependent expression not specific to petal but to leaf bud in *Arabidopsis*).

25 In addition to these promoters, other conventional promoters in plant field are usable.

(2) A method to suppress the function of ROT3 or CYP90D1:

It is possible to suppress functions of a specific gene by an antisense RNA method (a method for transducing an altered gene so as to be transcribed in reverse direction) or by a RNAi method (a method for transducing an altered gene so as to be ligated a part of a gene tandemly in forward and reverse and as to be transcribed throughout). The present invention applied the above-described method for

suppressing genetic expression. Since the target genes are CYP90D1 (SEQ ID: 1) and ROT3 (=CYP90C1, #51 to #1625 of SEQ ID NO: 3) are revealed, targeted suppression of their expression is possible.

5 (3) A method of combination:

It is prerequisite to prepare singly altered strains of CYP90D1 and ROT3 (=CYP90C1, #51 to #1625 of SEQ ID NO: 3), independently, then, two methods are possible: either crossing between them by classical genetics or direct transduction of both altered genes should bring about doubly altered strains.

10

(4) A method of precursor fermentation:

There are successful examples for at least a part of genes responsible to brassinosteroid biosynthesis showing enzymatic activity when these genes are expressed in yeast cells. Using the methods of these examples and providing appropriate precursors, it is possible to artificially synthesize castasterone or brassinolide (the final and active product) in yeast cells or in eucaryotic cells, wherein combination of ROT3 and CYO90D1 or one of the above-described genes are expressed.

20 Brief description of drawings:

Figure 1 shows the whole pathway of brassinosteroid biosynthesis.

Figure 2 shows morphology of leaves of wild type (Ws-2)(No. 1), a strain of reference example 1 (suppression of ROT3 function) (No.2), a strain of reference example 2 (suppression of both ROT3 and CYP90D1 function)(No. 3 and 4) of *Arabidopsis* cultivated in the same condition. No.3 and No.4 show a partly effective and a strongly effective strain, respectively.

Figure 3 shows morphology of leaves of strains without function of ROT3 and CYP90D1 after the treatment with intermediates of brassinosteroid synthesis and brassinolide. Control: no treatment, 6-D-CT: treated with (hereinafter the same) 30 6-Deoxocathasterone, 6-D-TE:6-Deoxoesterone, 6-D-3DT: 3-Dehydro-6-deoxoesterone, 6-D-TY: 6-Deoxotyphasterol, 6-D-CS: 6-Deoxocastasterone, CT: Cathasterone, TE: Teasterone, 3DT: 3-Dehydrotesterone,

TY: Typhasterol, CS: Castasterone, BL :Brassinolide.

Effects of the invention:

There are several practical defects in previous invention on the regulation of  
5 steps in biosynthetic pathway of steroid compounds, which show distinctive  
physiological activity in plants.

Namely, the regulatory factor of brassinosteroid biosynthesis previously  
elucidated is involved in the early steps of the biosynthetic pathway and enforced  
expression of the factor in transgenic plants brings about spindly growth of the whole  
10 plant and enlarges the plant. Therefore, there are no practical utility values for the  
above-described regulatory factor except for their occasional application. On the  
other hand, stopping a biosynthetic pathway in a transgenic plant resulted in  
miniaturization of the whole plant and, again, there are no practical utility except for  
their occasional application. In other words, conventional methods change the whole  
15 shape of a plant, which is practically not valuable. Practically usable and valuable  
transgenic plants are shown by the following examples: in horticulture, only the size  
of floral organs is large or only the size of leaves is small, or in improvement of  
vegetables, only the size of leaves is large. Therefore, conventional methods have  
difficulty in applying to biodesign of plants without the combination with a special  
20 expression-regulatory system. According to the present invention, in contrast to  
them, it becomes possible to control the size of a specific organ in a specific direction  
(specially in longitudinal direction) and the whole size of a plant by using the  
expression-regulatory system. According to the present invention, in contrast to  
them, it becomes possible to control the size of a specific organ in a specific direction  
25 (specially in longitudinal direction) and the whole size of a plant by using the  
combination of ROT3 (=CYP90C1) with CYP90D1.

Furthermore, the present invention elucidated that ROT3 (=CYP90C1) and  
25 CYP90D1 cooperatively regulate the final step of brassinosteroid biosynthesis.  
Therefore, the invention could be used for various industrial applications using as  
chemical synthesis of brassinosteriod.

The following examples illustrate this invention, however, it is not intended to  
30 limit the scope of the invention.

Manufacturing example 1.

As the strain knocked down the function of ROT3, the inventors used rot3-1 null mutant of *Arabidopsis* (Tsuge et al., Development 122: 1589-1600 (1996), a functional defect mutant of ROT3. The mutant cell line was seeded under sterilized conditions and cultured at 23°C under continuous illumination.

5

Manufacturing example 2.

To get the strain knocked down the function of both ROT3 and CYP90D1, the inventors, first of all, isolated cDNA of CYP90D1 from *Arabidopsis* using a primer set, ROT3h-cDNA-for: 5'-GTTAAAACACTAATGGACAC-3'(SEQ ID NO: 5);  
10 ROT3h-cDNA-rev: 5'-TGATTATATTCTTTGATCC-3'(SEQ ID NO: 6), which could specifically amplify the ROT3 homologue (CYP90D1). Then, the above-described CYP90D1 (SEQ ID NO: 1) clone was inserted to be transcribed in reverse direction from cauliflower mosaic virus 35S promoter into multipurpose vector pBI121, wherein hygromycin resistant gene was inserted as a selection marker and GUS protein coding region was removed. The construct was transduced into *Agrobacterium* (C58C1 Rif-resistant) and was introduced into *Arabidopsis* rot3-1 by in planta method, using conventional way of suspension culture medium of *Agrobacterium*. After the transformants were selected by hygromycin, transformants with homozygous inserted genes were isolated by self-pollination. Then, the strain was seeded under sterilized  
15 conditions and was cultured at 23°C under continuous illumination.  
20

Example 1

Figure 2 shows morphology of leaves of wild species (Ws-2)(No. 1), a strain of reference example 1 (suppression of RO3 function) (No.2), a strain of reference example 2 (suppression of both Rot3 and CYP90D1 function)(No. 3 and 4) of *Arabidopsis* cultivated in the same condition. While the leaves of the strain with suppressed function of ROT3 (Fig.2-2) are shorter in longitudinal direction compared to those of wild type (Fig.2-1), the leaves of the strain with suppressed function of both ROT3 and CYP90D1 (Fig2-3 & 4) are shorter further more than those of the above strains. In short, ROT3 and CYP90D1 are genes, which are cooperatively involved in biosynthetic pathway of brassinosteroi.

Example 2

The strain with suppressed function of both ROT3 and CYP90D1 prepared in reference example 2 was cultured from seeds in sterilized conditions. The seeds of the above-described strain were seeded on the MS medium (solidified by 0.2% Gelrite) supplemented with 2% (w/v) sucrose and were conventionally cultured at 23°C under continuous illumination after seeding under sterilized conditions.

On the other hand, aqueous solution (0.1 µM) of intermediates of brassinosteroid biosynthesis (e.g., 6-D-CT: 6-Deoxocathasterone, 6-D-TE: 6-Deoxoesterone, 6-D-3DT: 3-Dehydro-6-deoxoesterone , 6-D-TY: 6-Deoxotyphasterol , 6-D-CS: 10 6-Deoxocastasterone, CT: Cathasterone, TE:Teasterone, 3DT: 3-Dehydrotesterone, TY:Typhasterol, CS:Castasterone) and brassinolide (BL) (BL was obtained from WAKO pharmaceutical Co. (made by FujiKagaku Industry, Co.) and other brassinosteroids were gifts from Dr. Shouzou Jijioka, RIKEN and Dr. Tohide Takatsu, Jouetsu University of Education) was prepared.

15 The above-described plants (strains of suppressed function of both ROT3 and CYP90D1) were cultured in the above-described aqueous solution at the level of submersion under the solution with gently shaking. In the case of the treatment of leaves with the above-described intermediates or brassinolide, leafstalks were cut out by a surgical knife after taking out the plants under sterilized conditions and were 20 treated in the same way. Figure 3 shows the photographs of these leaves.

As shown in Fig.3, each brassinosteroid intermediate was not effective to plants without the function of ROT3 and CYP90D1, however, brassinolide (BL), the final product, induced large size of leaves and showed distinguished effects. Namely, ROT3 and CYP90D1 are cooperatively involved in the synthesis of brassinolide, the 25 final product of the biosynthetic pathway of brassinosteroids.

Example 3.

The concentrations of brassinosteroids were determined in wild strains (Ws-2), the strain of reference example 1 (rot3-1 and rot3-5, suppressed function of ROT3) 30 and the strain of reference example 3 (rot3/CYP90D1, suppressed function of ROT3 and CYP90D1) of *Arabidopsis*. The concentration was determined by harvesting the ground part of the plants at the time of rosette formation by reaping, by freezing

and drying, and by detecting using HPLC and GC-MS. The results are shown in Table 1.

Table 1

	ng/g			
	Ws-2	rot3-1	rot3-5	rot3/CYP90D1
6-Deoxoesterone	0.05	0.19	0.11	0.26
3-Deoxotyphasterol	2.30	3.49	4.30	0.38
6-Deoxocastasterone	2.60	1.88	4.00	0.034
Teasterone	-	0.004	0.02	-
Typhasterol	0.27	0.38	0.46	0.014
Castasterone	0.28	0.31	0.50	0.020
Brassinolide	0.20	0.04	0.06	-

5 Note: (-) in the table shows that the value is less than the limit of detection (0.001 ng/g).

As shown in the table, in the strain (rot3-1 and rot3-5) with suppressed ROT3, the production of brassinolide decreased remarkably, as a consequence of this, the 10 production of brassinosteroids in the previous stage (especially castasterone) increased. However, suppression of ROT3 did not completely block the production of brassinolide. In other words, ROT3 itself dose not completely regulate the biosynthesis of brassinolide.

On the other hand, in the strain (rot3/CYP90D1) with suppressed function of 15 both ROT3 and CYP90D1, the amount of brassinolide extremely decreased among intermediates of brassionosteroid biosynthesis, which indicates that the pathway of the synthesis of brassinolide from castasterone is completely blocked by simultaneous suppression of both ROT3 and CYP90D1. In other words, the production of brassinolide is completely regulated by the combined action of ROT3 and CYP90D1.